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Short Communication

Evaluation of microbial strains for linoleic acid hydroxylation and reclassification of strain ALA2

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Abstract

In previous studies, a new microbial strain ALA2 was isolated which produced many new products from linoleic acid [Gardner H.W., Hou C.T., Weisleder D. and Brown W. 2000. Lipids 35: 1055–1060; Hou C.T. 1998. 12,13,17-Trihydroxy-9(*Z*)-Octodecenoic acid and derivatives and microbial isolate for production of the acid. US Patent No. 5, 852, 196]. Strain ALA2 was preliminary identified as *Clavibacter* sp. based on its physiological and fatty acid profiles. To determine if strain ALA2 is the optimal strain for industrial applications, other related strains were screened for their abilities to convert linoleic acids. Two strains from *Clavibacter* and 20 type strains from the phylogenetically related genus *Microbacterium* were studied. Surprisingly, all of these strains tested showed very little or no activity in converting linoleic acid. On reexamination of the identification of strain ALA2, the sequence of the 16S ribosomal RNA gene of ALA2 was found to be 99% identical to that of *Bacillus megaterium* and the strain was also found to have 76.3% DNA homology to the *B. megaterium* type strain. Therefore, strain ALA2 is now reclassified as *B. megaterium*. Screening of 56 strains of *B megaterium* strains showed that many of them were able to produce reasonable amounts of hydroxyl fatty acids from linoleic acid, although strain ALA2 possessed the greatest activity.

It has been reported that microbial systems convert unsaturated fatty acids to monohydroxy-, dihydroxy- and trihydroxy-fatty acids (Hou 1995a; Hou and Bagby 1991; Wallen et al. 1962). It is feasible to produce various value-added hydroxy fatty acids and their derivatives for industrial applications by using the unique reaction specificities of microbial enzymes. Earlier, we isolated a microbial culture strain ALA2 from a soil sample collected from McCalla, Alabama. Comparison with known strains using the Biolog System indicated that strain ALA2 had the characteristics of the genus *Clavibacter*. Therefore, it was preliminary designated as *Clavibacter* sp. ALA2 (Hou et al. 1997). Strain ALA2 is a unique microbe, which produces a variety of hydroxy fatty acids from linoleic acid (Gardner et al. 2000; Hou 2003). Hydroxy fatty acids can be used not only as speciality chemicals, but also as bioactive agents such as antifungal agents (Kato et al. 1984; Masui et al.

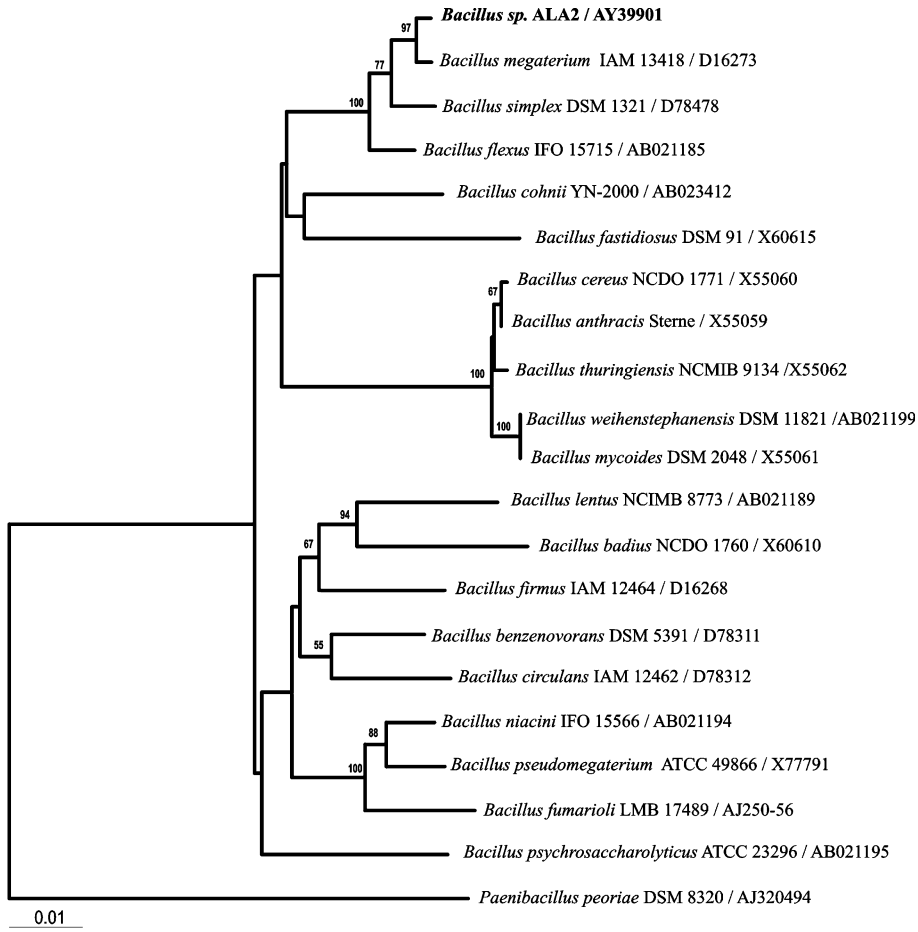

Figure 1. Phylogenetic tree of the *Bacillus* species closest to ALA2 calculated from 16S rDNA sequences using Kimura's evolutionary distance method (Kimura 1980) and the neighbor-joining method (Saitou and Nei 1987). Bar marker scale equals 0.01 nucleotide substitutions per site.

1989). The main product, 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA) was found to inhibit the growth of plant pathogenic fungi (Hou 1998; Hou and Forman 2000). Moreover, the structure of the tetrahydrofuranyl fatty acids (Hosokawa et al. 2003a,b) produced resemble those of known anticancer agents (Kawagishi et al. 1990; Stadler et al. 1994). Diepoxy bicyclic fatty acid products from linoleic acid are new chemical entities with many functional groups in their molecules. Applications in the biomedical and speciality chemical industry are anticipated.

To develop an industrial process for the production of these hydroxy fatty acids, it is important to make sure that strain ALA2 is the best strain within its related group. Since our ARS Culture Collection is one of the largest in the world, we screened members of the genus *Clavibacter* and the phylogenetically related genus *Microbacterium*. All of the *Clavibacter* strains in ARS Culture Collection (only two strains) and 20 *Microbacterium* strains (one strain from each species) were studied for their abilities to convert linoleic acids. Surprisingly, most showed no activity in converting linoleic acid to 12,13,17-THOA except four strains (indicated * in the following list) which possessed slight activity (less than 2% conversion) as follows: *Clavibacter* (**C. michiganense* NRRL B-33, and *Clavibacter* sp. NRRL B-23013) and *Microbacterium* (*M. aurum* NRRL B-24210, *M. esteraromaticum* NRRL B-24213, *M. arabinogalactanolyticum* NRRL B-24215, *M. aurantiacum* NRRL B-24217,

**M. chocolatum* NRRL B-24218, *M. foliorum* NRRL B-24224, *M. aerolatum* NRRL B-24228, **M. barkeri* NRRL B-24231, *M. arborescens* NRRL B-24239, *M. dextranolyticum* NRRL B-23242, *M. keratanolyticum* NRRL B-24211, **M. halophilum* NRRL B-24219, *M. hominis* NRRL B-24220, *M. ketosireducens* NRRL B-24221, *M. kitamiense* NRRL B-24226, *M. laevaniformans* NRRL B-24229, *M. lacticum* NRRL B-24233, *M. imperiale* NRRL B-24235, *M. liquefaciens* NRRL B-24237 and *M. gubbeenense* NRRL B-24242). These findings prompted us to re-examine strain ALA2. Strain ALA2 was observed microscopically to consist of large rods (approximately 1.2 μm in diameter) containing refractile round to ovoid spores, typical of a few species of the genus *Bacillus* including *B. megaterium* (Krieg 1984). The sequence of the 16S rRNA gene was determined following previously described procedures (Labeda and Kroppenstedt 2000) and deposited in Gene-Bank under Accession No. AY739901 and the phylogenetic position of the strain within the genus *Bacillus* was determined (Figure 1). ALA2 was found to be mostly closely related (99% 16S rRNA sequence similarity) to *B. megaterium*. The DNA relatedness (Kimura 1980; Saitou and Nei 1987) between strain ALA2 and the type strain of *B. megaterium* NRRL B-3712 was found to be 76.3% based on triplicate determinations. A comparison of the physiological properties of strain ALA2 with *B. megaterium* NRRL B-3712^{T} using the API 50 CH and ZYM test strips (bioMèrieux, Inc., Durham, NC) according to the instructions from the manufacturer it was found that there were only minor differences between the strains. NRRL B-3712 utilized D-xylose, D-mannose, methyl-α-D-glucopyranoside, and inulin and had an active naphthol-AS-BI-phosphorylase while strain ALA2 did not. Additionally, strain ALA2 had an active acid phosphatase, α-galactosidase, and α-glucosidase while NRRL B-3712 did not. These data provide evidence that ALA2 represents a strain of the species *B. megaterium*. Therefore, strain ALA2 is reclassified as *B. megaterium* ALA2.

Subsequently, we screened 56 other *B. megaterium* strains for their ability to convert linoleic acid to 12,13,17-THOA (Hou 1996). We found that 36 of them were able to produce reasonable amount of hydroxyl fatty acids from linoleic acid (Table 1). However, strain ALA2 possessed the greatest activity.

Table 1. Screening of *Bacillus megaterium* strains for conversion of linoleic acid to trihydroxy fatty acid.

Microbial strains	Activity (THOAs mg/50 ml)
Bacillus megaterium ALA2	46.72
B. megaterium NRRL B-14398	29.78
B. megaterium NRRL B-353	25.95
B. megaterium NRRL BD-238	24.58
B. megaterium NRRL BD-270	23.93
B. megaterium NRRL BD-251	23.76
B. megaterium NRRL B-350	23.65
B. megaterium NRRL BD-246	22.40
B. megaterium NRRL B-1372	21.78
B. megaterium NRRL BD-262	21.59
B. megaterium NRRL B-1827	20.73
B. megaterium NRRL BD-237	19.15
B. megaterium NRRL BD-250	17.64
B. megaterium NRRL B-1371	16.11
B. megaterium NRRL B-42	15.50
B. megaterium NRRL BD-252	15.13
B. megaterium NRRL B-1369	14.70
B. megaterium NRRL B-3254	14.58
B. megaterium NRRL B-1366	13.90
B. megaterium NRRL BD-271	13.90
B. megaterium NRRL B-23938	13.02
B. megaterium NRRL B-23940	12.71
B. megaterium NRRL B-3256	8.25
B. megaterium NRRL B-1368	7.25
B. megaterium NRRL B-14308	7.02
B. megaterium NRRL BD-254	6.86
B. megaterium NRRL B-1851	6.62
B. megaterium NRRL BD-26	6.46
B. megaterium NRRL B-283	5.51
B. megaterium NRRL B-349	4.93
B. megaterium NRRL BD-236	4.37

The inactive *B. megatherium* strains are: NRRL B-351, B-352, B-938, B-1369, B-1370, B-3437, B-3440, B-3442, B-3701, B-3703, B-3764, B-4272, B-14007, B-14147, BD-235, BD-242, BD-243, BD-244, BD-245, BD-249, BD-255, BD-257, BD-261, BD-263, BD-265, BD-3694.

*NRRL – Northern Regional Research Laboratory (Current name, National Center for Agricultural Utilization Research), ARS, USDA.

From the literature, we note that *Bacillus sphaericus*-like organisms convert fatty acids to keto acids (Kuo et al. 2002). The conversion of oleic acid to ketostearic acid was carried out by hydratase and secondary alcohol dehydrogenase, as described earlier (Hou 1995b). The enzymes that convert ricinoleic acid and linoleic acid to unknown new products were not defined but the same enzyme reaction mechanism possibly occurs, as judged from the GC retention times of the products. However, the enzyme systems involved in the production of oxygenated products from linoleic acid by *B. megaterium* strains are more complex, possibly involving oxygenases and not hydratase type enzymes. Self-sufficient cytochrome P450 monooxygenase from *B. megaterium* (CYP102A1) has been well studied (Narhi and Fulco 1986; Wen and Fulco 1987). Two genes CYP102A2 and CYP102A3 from *B. subtilis* code for single-peptide monooxygenases, comprising both a heme and a FAD/FMN-containing reductase domain and having a notable sequence similarity to CYP102A1 (Kunst et al. 1997; Budde et al. 2005). CYP102A3 is involved in the hydroxylation of unsaturated, saturated and branched-chain fatty acids (Gustafsson et al. 2001; Lentz et al. 2004). Therefore, it would be interesting to compare the fatty acid bioconversion ability and their reaction products between *B. subtilis* and *B. megaterium* strains.

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